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translator to RWS Group plc, of Europa House, Marsham Way, Gerrards Cross, Buckinghamshire, England declare;

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2. That I am well acquainted with the French and English languages.

3. That the attached is, to the best of my knowledge and belief, a true translation into the English language of the accompanying copy of the specification filed with the application for a patent in France on 9 February 2004 under the number 10/774,721 and the official certificate attached hereto.

4. That I believe that all statements made herein of my own knowledge are true and that all statements made on information and belief are true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the patent application in the United States of America or any patent issuing thereon.

E. Parrish

For and on behalf of RWS Group plc

The 5th day of April 2004

OLIGONUCLEOTIDES WHICH INHIBIT EXPRESSION OF THE OB-RGRP PROTEIN AND METHOD FOR DETECTING COMPOUNDS WHICH MODIFY THE INTERACTION BETWEEN PROTEINS OF THE OB-RGRP FAMILY AND THE LEPTIN RECEPTOR

The present application relates to oligonucleotides which inhibit expression of the OB-RGRP protein and to uses thereof for preventing and/or treating leptin-related pathological conditions.

It also relates to a method for detecting leptin receptor ligands using the energy transfer between, firstly, fusion proteins composed of leptin receptors and of energy-donor or -acceptor proteins and, secondly, fusion proteins composed of OB-RGRP or of MYO47 and of energy-donor or -acceptor proteins.

It also relates to fusion proteins for implementing this method.

Leptin is a 16 kDa protein secreted mainly by the adipose tissue, which binds to a receptor (OB-R) belonging to the cytokine receptor family. Five membrane-bound isoforms of this receptor have been identified, and derive from alternative splicing of the same gene. These isoforms which have the same extracellular and transmembrane domain are characterized by intracellular domains of varying sizes (Tartaglia et al (1995) Cell 83, 1263-1271). A soluble form of the receptor has also been identified and comes from an alternative splicing or a proteolytic cleavage of the extracellular domain of the membrane-bound forms. The short form of the receptor (OB-Rs), which appears to be involved in transporting leptin across the blood-brain barrier, is the most expressed isoform. The long form (OB-Rl) is only expressed in a few tissues, such as the hypothalmus, and appears to be responsible for most of the biological effects of leptin (Sweeney, G. (2002) Cell Signal 14, 655-663). Leptin and its receptor have been the subject of particular attention due to their involvement in the regulation of energy balance and of the metabolism, and in the neuroendocrine response to food intake. Recently, it has been shown that leptin is also involved in important addition functions, such as regulation of the bone mass, angiogenesis, cicatrization, thrombus formation, sexual maturation, hematopoiesis, the regulation of immunity and inflammation, fetal development and cancer. The administration of leptin to leptin-deficient organisms such as mice (ob/ob) and certain humans causes a decrease in the lipid mass in various tissues, such as the liver and the adipose tissue (Halaas et al. (1995) Science 269, 543-546, Pelleymounter et al. (1995) Science 269, 540-543, Campfield et al. (1995) Science 269,

546-549, Farooqi et al. (1999) N Engl J Med 341, 879-884). This treatment with leptin also improves the sensitivity to insulin and decreases the fatty mass in mice and humans exhibiting lipodistrophy (Shimomura et al. (1999) Nature 401, 73-76, Oral et al. (2002) New England Journal of Medicine 346, 570-578, Petersen et al. (2002) J Clin Invest 109, 1345-1350). Obese individuals are generally resistant to leptin. The reasons for this resistance are still poorly understood, but several mechanisms have been suggested: a deficiency in leptin transport across the blood-brain barrier, a deficiency in activation of OB-R or in the signaling by these receptors, and the overexpression of negative regulators such as SOCS3 and PTP-1B (Bjorbaek et al. (2000) J Biol Chem 275, 40649-40657, Cheng et al. (2002) Developmental Cell 2, 497-503, Cook and Unger (2002) Developmental Cell 2, 385-387). Understanding the mechanisms of resistance to leptin requires a more detailed characterization of the mechanisms involved in OB-R activation.

OB-R is constitutively associated with janus kinase 2 (JAK 2). The binding of JAK2 to the receptor is critical for the signaling by OB-R and has been proposed as being involved in stabilizing the OB-R receptor dimers. Activation by agonists is thought to cause a change in conformation in the juxtamembrane region of the cytoplasm tail of the OB-R. JAK2, which is constitutively linked to the box1 motif in this region, is activated by autophosphorylation and then phosphorylates the OB-RI receptor but not the OB-Rs receptor. The phosphorylation of OB-RI allows anchoring of STAT proteins, which bind to the receptor and are activated by phosphorylation of tyrosine. The activated STAT proteins dimerize and translocate into the nucleus in order to stimulate the transcription of genes via STAT response elements (Tartaglia (1997) J Biol Chem 272, 6093-6096).

Recently, a second promoter for the leptin receptor has been discovered. Interestingly, a second transcript is co-expressed with the OB-R messengers from this promoter. This transcript has been observed in several species, such as mice, rats, humans, yeast and *C. elegans* (Bailleul et al. (1997) Nucleic Acids Res 25, 2752-2758). *In situ* hybridization experiments confirm the coexpression of OB-R and of the associated gene in the brain of mice, including the hypothalamic regions involved in regulating body weight (Mercer et al., J Neuroendocrinol 2000 July; 12(7):649-55). The corresponding protein is composed of 131 amino acids and is called OB-R-gene related protein (OB-RGRP). This protein was the subject of patent application WO 98/05792.

The fact that OB-RGRP is expressed in yeast and nematodes, which are organisms lacking leptin receptors, indicates a more general role for OB-RGRP, supported by the deletion of this protein in yeast which causes a deficiency in transport of proteins from the golgi to the vacuoles (Belgareh-Touze et al. (2002) Molecular Biology Of The Cell 13, 1694-1708).

In 2002, a cDNA called MY047 was cloned from a human brain cDNA library (Huang et al. (2001) Biochimica et Biophysica acta. Gene structure and expression 327-331). This protein is also the subject of application EP 0 969 091. The function of the corresponding protein is still unknown. MY047 exhibits 68% homology with OB-RGRP, suggesting that these two proteins belong to the same family. Analysis of the sequences available for the human genome sequencing project shows that no other homolog exists.

The applicants have endeavored to determine the role of OB-RGRP and its relationships with leptin receptors.

They have thus shown the specificity of the interactions between OB-RGRP and the OBRs receptor.

They have also shown that it is possible to specifically modify the expression of leptin receptors at the cell surface using oligonucleotides directed against the leptin receptor gene associated protein (OB-RGRP).

A subject of the present application is therefore optionally modified oligonucleotides comprising from 8 to 50 nucleotides which hybridize specifically with the sequence SEQ ID No. 1 and which inhibit OB-RGRP expression.

Advantageously, these oligonucleotides promote the expression of leptin receptors at the cell surface.

Preferentially, these oligonucleotides are antisense oligonucleotides.

Preferentially, these oligonucleotides comprise a sequence exhibiting at least 60%, 70%, 80% or 90% identity with the sequence SEQ ID No. 2.

According to an advantageous embodiment, in these oligonucleotides, nucleotides are thioesterified.

According to another advantageous embodiment, in these oligonucleotides, nucleotides are 2'-O-methylated.

According to another advantageous embodiment, these oligonucleotides have a triethylene glycol residue at their 3' ends.

Although the most commonly used form of antisense compounds is in the form of antisense oligonucleotides, the present invention includes oligonucleotide derivatives and compounds which mimic their structure, such as those described hereinafter, without this list being limiting. The antisense compounds in agreement with this invention preferably comprise from 8 to 50 nucleobases (i.e. they are oligomers made up of 8 to 50 nucleotide units). The antisense compounds particularly targeted are antisense oligonucleotides, more specially those which are made up of approximately 12 to 30 nucleobases. The antisense compounds comprise ribozymes, oligozymes or other short catalytic RNAs or catalytic oligonucleotides which hybridize with the target nucleic acid and modulate its expression. A nucleoside is a combination of a nitrogenous base and a sugar. The base of a nucleoside is generally a heterocyclic nitrogenous base. The two most common types of heterocyclic base are purine and pyrimidine bases. The nucleotides are nucleosides which carry a phosphate group covalently bonded to the sugar of the nucleoside. For the nucleosides comprising a pentanofuranose, the phosphate may be bonded to the hydroxyl at position 2', 3' or 5' of the sugar. The formation of nucleotides comes from the covalent attachment of the phosphate group to two adjacent nucleosides, which makes it possible, step by step, to obtain a linear oligomer. The two ends of such a linear polymer can, in turn, join together to form a circular structure, but the open structure is generally preferred. In the nucleotide structure, the phosphate groups are considered to form the internucleoside skeleton of the oligonucleotide. The normal bond in the RNA or DNA skeleton is a 3'-5' phosphodiester linkage. Specific examples of antisense compounds which can be used in this invention include oligonucleotides containing a modified backbone or unnatural internucleoside bonds. Thus, oligonucleotides with a modified backbone comprise those which conserve a phosphate atom in their skeleton and those which are lacking therein. For the needs of the present invention, modified oligonucleotides which do not have a phosphorus atom in their internucleoside bond can, nevertheless, be considered to be oligonucleotides. The backbone of these modified oligonucleotides may comprise, for example, the following groups: phosphorothioates, chiral phosphorothioates, phosphorodithioates, phosphotriesters, aminoalkylphosphotriesters, methyl and other alkyl phosphonates,

including 3'-alkylene phosphonates, 5'-alkylene phosphonates and chiral phosphonates, phosphinates, phosphoramidates, including 3'-aminophosphoramidate and aminoalkylphosphoramidates, thionophosphoramidates, thionoalkylphosphonates, thionoalkylphosphotriesters, selenophosphates and borophosphates which form normal 3'-5' bonds, and analogs thereof which form 2'-5' bonds, and also those which exhibit a reverse polarity, i.e. comprising at least one internucleoside bond of the 3'-3', 5'-5' or 2'-2' type. The form of oligonucleotides having a reverse polarity which is preferentially used is that which has the first internucleoside bond in 3' is of the 3'-3' type. This corresponds to a single inverted nucleotide residue which may, moreover, be abasic, i.e. in which the heterocyclic nitrogenous base is missing or replaced with a hydroxyl group. The various forms (saline or free acid) are included in the field of this invention.

The backbone of the modified oligonucleotides lacking a phosphorus atom is preferentially made up of short alkyl or cycloalkyl chains, including derivatives thereof comprising one or more hetero atoms, acting as an internucleoside bond. This type of backbone may be based on a morpholino bond (partly consisting of the sugar of the nucleoside), on siloxane, on formacetyl and thioformacetyl, on methylene formacetyl and methylene thioformacetyl, on riboacetyl, on alkenes, on sulfamates, on sulfonate and sulfonamide, on methyleneimine and methylene hydrazine, on amide, and on any other group comprising various nitrogen, sulfur and oxygen atoms or methyl groups.

For other oligonucleotide analogs, the sugar and the internucleoside bond (i.e. the backbone) are replaced at the same time in the nucleotide structure with new groups. The heterocyclic nitrogenous base is conserved in order to ensure hybridization with the target nucleic acid. Such oligomeric compounds, PNAs (for Peptide Nucleic Acids), have shown an excellent capacity for hybridization. In these compounds, the skeleton of the oligonucleotide is replaced with an amide-based backbone, in particular with aminoethyl glycine, grafted directly or indirectly onto the nitrogenous bases. In addition, thorough teaching regarding these PNAs may be found in Nielsen et al., Science, 1991, 254, 1497.

The invention incorporates more particularly oligonucleotides with a phosphorothioate, amide and morpholine backbone, and the oligonucleotides with a hetero atom skeleton, more precisely:
$-CH_2-NH-O-CH_2-$

$-CH_2-N(CH_3)-O-CH_2-$ (called methylene(methylimino) or MMI skeleton)
$-CH_2-O-N(CH_3)-CH_2-$
$-CH_2-N(CH_3)-N(CH_3)-CH_2-$
$-O-N(CH_3)-CH_2-CH_2-$ (in which the phosphodiester bridge is: O-P-O-CH_2).

The modification of the oligonucleotides may also be carried on the sugars: the preferred substitutions are at position 2' (F; O-, N- or S-alkane, O-, N- or S-alkene or O-, N- or S-alkyne derivatives of length C1 to C11, which may or may not be substituted) in particular, the preferred derivatives are:
$O-[(CH_2)nO]m-CH_3$
$O-(CH_2)n-O-CH_3$
$O-(CH_2)n-NH_2$
$O-(CH_2)n-CH_3$
$O-(CH_2)n-O-NH_2$
$O-(CH_2)n-O-N[(CH_2)n-CH_3]_2$
in which n and m range from 1 to 10.
Other modifications of the 2' position include the following groups: aliphatic chains, which may or may not be substituted, of length C1 to C10, aryl chains, aryl-alkyl chains and alkyl-aryl chains; -SH, $-SCH_3$, -OCN, -Cl, -Br, -CN, CF_3, $-OCF_3$, $-SO_2CH_3$, $-ONO_2$, $-NO_2$, $-N_3$, $-NH_2$; substituted silyls; "reporter" groups; intercalating groups; RNA cleavage groups; group to improve the pharmacodynamic capacities of an oligonucleotide. The preferred modifications include the groups:
2'-methoxyethoxy (2'-O-$CH_2CH_2OCH_3$, also called 2'-O-(2-methoxyethyl) or 2'-MOE) (Martin et al., Helv. Chim. Acta, 1995, 78f 486-504) 2'-dimethylaminooxyethoxy ($O(CH_2)_2ON(CH_3)_2$, also called 2'-DMAOE;
2'-dimethylaminoethoxyethoxy (2'-O-$CH_2OCH_2-N(CH_2)_2$, also called 2'-dimethylaminoethoxyethyl or 2'-DMAEOE).
Another advantageous modification leads to the formation of LNAs (Locked Nucleic Acids) in which the hydroxyl at position 2' is attached to the carbon at position 3' or 4' of the sugar, then forming a sugar with a bicyclic structure. The preferred bridging occurs via a methyl or ethyl linkage between the 2' oxygen and the 4' carbon.
Other preferred substitutions at position 2' include:
$-O-CH_3$ (2'-methoxy)
$-O-(CH_2)_3-NH_2$ (2'-aminopropoxy)
$-CH_2-CH=CH_2$ (2'-allyl)
$-O-CH_2-CH=CH_2$ (2'-O-allyl)
-F (2'-fluoro).

These modifications at 2' may be in the ribo (lower) or arabino (upper) position. The 2'-fluoro substituent is the preferred one in the arabino position.

Similar modifications may be made on other positions, in particular at position 3' of the sugar of the nucleotide at the 3'-terminal end or in the oligonucleotides with a 2'-5' backbone, and at position 5' of the sugar at the 5'-terminal end. The sugars of the oligonucleotides can also be replaced with analogs (for example a cyclobutyl can be substituted for a pentofuranyl).

The oligonucleotides can also comprise modifications or substitutions on the nucleobases (nitrogenous heterocyclic bases called "bases" by those skilled in the art). The natural (unmodified) bases are purines (adenine A and guanine G) and pyrimidines (cytosine C, thymine T and uracil U). Included among the modified bases are natural or synthetic molecules such as 5-methylcytosine, 5-hydroxymethylcytosine, xanthine, hypoxanthine, 2-aminoadenine; 6-methyl, 2-methyl and other alkyl derivatives of purine bases (A and G); 2-thio derivative (C, T and U); 5-halo derivative (U, C); 5-propynyl cytosine derivative (U and C); 6-azo derivative (U, T and C); 5-uracil; 4-thiouracil; 8-halo, 8-amino, 8-thiol, 8-thioalkyl, 8-hydroxyl and other adenines and guanine substituted at position 8; 5-halo (in particular 5-bromo), 5-trifluoromethyl and other uracils and cytosines substituted in position 5; 7-methylguanine and 7-methyladenine; 2-fluoroadenine; 2-aminoadenine; 8-azaguanine and 8-azaadenine; 7-deazaguanine and 7-deazaadenine; 3-deazaguanine and 3-deazaadenine. In the other modified bases, tricyclic pyrimidines are found such as phenoxazine cytidine (1H-pyrimido[5,4-b][1,4]benzoxazin-2-(3H)-one), phenothiazine cytidine (1H-pyrimido[5,4-b][1,4]benzothiazin-2(3H)-one), substituted phenoxazine cytidine (such as 9-(2-aminoethoxy)-H-pyrimido[5,4-b][1,4]benzoxazine-2(3H)-one), or carbazole cytidine (2H-pyrimido[4,5-b]indol-'2-one).

The modified bases comprise the compounds in which the purine or pyrimidine heterocycle is replaced with another heterocycle, for example 7-deazaadenine, 7-deazaguanosine, 2-aminopyridine or 2-pyridone (The Concise Encyclopedia Of Polymer Science And Engineering, pages 858-859, Kroschwitz, J.I., ed. John Wiley & Sons, 1990; Englisch et al., Angewandte Chemie, International Edition, 1991, 30, 613; Sanghvi, Y.S., Chapter 15, Antisense Research and Applications, pages 289-302, Crooke, S.T. and Lebleu, B., ed., CRC Press, 1993). Some of these modified bases may be of great value for increasing the affinity of the oligomeric

compounds of the invention, such as pyrimidines substituted at position 5, azapyrimidines, or N- and O-substituted purines (such as 2-aminopropyladenine, 5-propynyl uracil, 5-propynyl cytosine). The substituted 5-methylcytosines have a positive effect on the stability of oligomer-nucleic acid duplexes (Sanghvi, Y.S., Crooke, S.T. and Lebleu, B., eds., Antisense Research and Applications, CRC Press, Boca Raton, 1993, pp. 276-278) and are the preferred substitution, in particular in combination with 2'-methoxyethyl modifications of the sugars.

Preferentially, these oligonucleotides are in the single-stranded form.

According to a particularly advantageous embodiment, these oligonucleotides comprise a sequence exhibiting at least 60%, preferably 70%, 80% or 90%, identity with the sequence SEQ ID No. 2, in which the nucleotides at positions 2, 4, 6, 7, 9, 11, 13, 15, 17, 19 and 20, in the 5' to 3' direction, are thioesterified.

According to a particularly advantageous embodiment, these oligonucleotides comprise a sequence exhibiting at least 60% identity with the sequence SEQ ID No. 2, in which the nucleotides at positions 1, 2, 3, 4, 5, 16, 17, 18, 19 and/or 20, in the 5' to 3' direction, are 2'-O-methylated.

Preferentially, the oligonucleotides according to the present invention are DNAs.

A subject of the present invention is also oligonucleotides of the iRNA (Interfering Ribonucleic Acid) type comprising from 10 to 60 nucleotides, and preferably from 15 to 25 nucleotides, which hybridize specifically to the sequence SEQ ID No. 21 and which inhibit the expression of OB-RGRP.
Preferentially, such iRNAs comprise 17 or 19 nucleotides taken continuously from the sequence SEQ ID No. 21, or from the sequence complementary thereto.
These iRNAs may be double-stranded, in which case they advantageously consist of two strands comprising from 15 to 60 nucleotides. According to a preferred embodiment, such an iRNA is one in which at least one of the two strands comprises a sequence exhibiting at least 60%, preferably 70%, 80% or 90%, identity with one of the sequences SEQ ID No. 37 or SEQ ID No. 38.

The iRNAs may also be expressed in single-stranded form. Such iRNAs may then comprise a loop. They advantageously comprise from 15 to 60 nucleotides. According to a preferred embodiment, such an iRNA comprises a sequence exhibiting at least 60%, preferably 70%, 80% or 90%, identity with the sequence SEQ ID No. 42.

Nucleotides A(A/G) and (C/T)T can be added respectively in 5' and in 3' of this sequence of 17 or 19 nucleotides. Other types of residues or chemical groups can, however, be added to these two ends, provided that they do not decrease the activity of the antisenses.

The nucleotide modifications described for the antisenses are also possible for those making up the composition of the siRNAs.

The present invention also includes any modifications of the antisenses or of the iRNAs which are directed toward increasing the resistance of these compounds to cellular nucleases, or their penetration into cells and/or their effectiveness in targeting the OB-RGRP sequence.

When they are DNAs, the oligonucleotides according to the present invention can be produced conveniently and routinely by the well-known technique of solid-phase synthesis. The equipment for such synthesis is sold by various specialized companies, such as Applied Biosystems (Foster City, CA). The synthesis of the antisenses in the present invention makes use of chemical synthesis on a suitable support according to methods known to those skilled in the art, in particular described by E. Uhlmann, A. Peyman, A. Ryte, A. Schmidt and E. Buddecke (1999, Methods in Enzymology 313: 268-284) and by E. Uhlmann (Recent advances in the medicinal chemistry of antisense oligonucleotides, Current Opinion of Drug Discovery and Development 3: 203-213, 2000). Any other method of synthesis known to those skilled in the art may also be used.

When they are iRNAs, the oligonucleotides according to the present invention can be synthesized by chemical synthesis, when they are synthetic iRNAs, or expressed in situ using vectors for synthesizing such oligonucleotides, or obtained by in vitro cleavage of a double-stranded RNA with RNAse III or the DICER enzyme.

siRNAs (small iRNAs) can be obtained from various suppliers, such as Proligo (Proligo France SAS 1 rue Robert et Sonia Delaunay 75011 Paris) Dharmacon (Dharmacon, Inc. 1376 Miners Drive #101 Lafayette, CO 80026) and Ambion (Ambion (Europe) Ltd. Ermine Business Park Spitfire Close Huntingdon, Cambridgeshire PE29 6XY United Kingdom), or can be

synthesized using kits marketed by various companies, such as Dharmacon and Ambion.
Preferentially, the iRNAs according to the present invention are in double-stranded form.

After synthesis, the iRNAs are first of all taken up in RNAse-free water. The pairing of the two single-stranded molecules can be carried out as follows: 20 $\mu mol.L^{-1}$ of each strand are mixed in the pairing buffer (100 $mmol.L^{-1}$ of potassium acetate, 30 $mmol.\ L^{-1}$ of HEPES-KOH, pH 7.4, 2 $mmol.L^{-1}$ of magnesium acetate) and then heated at 90°C for 1 min, followed by incubation for 1 h at 37°C.
Transfection of the siRNAs can be carried out using the same protocol as for transfection of the antisenses.

An alternative for the iRNA is the use of vectors which allow synthesis of antisense RNAs specific for the gene to be silenced and which will pair in the transfected cells to give an siRNA. A first vector system allows expression of an antisense sequence by two promoters in opposite direction, on each side of this sequence, producing two complementary RNAs which will pair in the transfected cells and give an siRNA. Another vector system uses the synthesis of an RNA having the sequence of the antisense followed by the sense sequence, a few nucleotides apart, which will create a stem-loop RNA structure which will be cleaved in the transfected cells to give an siRNA. Yet another vector system involves the expression of a double-stranded RNA, up to 600 base pairs long, which cannot leave the nucleus since it does not have the necessary sequences: no 3′-cap (ribozyme site) nor a 5′-poly-A tail (MAZ zinc finger protein binding site). This long RNA is cleaved in the nucleus to give functional siRNAs which will pass into the cytoplasm and cause degradation of the target RNA.
These vectors are transfected conventionally as described above for the various DNAs. Stable lines which exhibit a knockout of the target gene can be obtained by antibiotic selection conventionally used to obtain lines.

In general, those skilled in the art may refer, for the iRNAs to the following publications: Elbashir S.M. et al. (2001, *Nature* **411**: 494-498), Elbashir S.M. Lendeckel W. and Tuschl T. (2001, *Genes & Dev.* **15**: 188-200) and Masters J.R., et al. (2001. *Proc. Natl. Acad. Sci. USA* **98**: 8012-8017).

Vectors which allow the expression of iRNAs can be obtained as described by Brummelkamp T.R., Bernards R., Agami R. (2002. *Science* **296**: 550-553) and Yu J.Y., DeRuiter S.L., and Turner D. (2002., *Proc. Natl. Acad. Sci. USA* **99**: 6047-6052) and Shinagawa T. and Ishii S. (2003, *Genes & Dev.* **17**: 1340-1345).

Such vectors, and also cells containing such vectors, are subjects of the present application.

A subject of the present invention is also medicinal products containing such oligonucleotides, vectors and cells, and pharmaceutical compositions containing a pharmacologically active amount of such oligonucleotides, vectors and cells and pharmaceutically acceptable excipients.

Another subject of the present invention is the use of such oligonucleotides, vectors and cells, for producing a medicinal product for preventing and/or treating leptin-related pathological conditions.

A subject of the invention is also a method of curative or preventive treatment of leptin-related diseases, consisting in administering such oligonucleotides, vectors and cells to a patient suffering from said disease.

Another subject of the invention is a method for determining the modification, by a compound, of the interaction between the OB-RGRP or the MYO47 protein, or a protein exhibiting at least 65% identity with this protein or with the MYO47 protein, and the leptin receptor.

It also relates to fusion proteins for implementing this method, and also to nucleic acids encoding these proteins.

A subject of the invention is also a method of curative or preventive treatment of leptin-related diseases, consisting in administering a ligand selected using the method defined above to a patient suffering from said disease.

A first subject of the present invention is therefore a fusion protein which is composed of a sequence exhibiting at least 65% identity with the sequence SEQ ID No. 4, or the sequence SEQ ID No. 16, or of a substantial part of the sequence SEQ ID No. 4 or of the sequence SEQ ID No. 16, and of an

energy-donor or energy-acceptor protein, or of a substantial and active part of an energy-donor or energy-acceptor protein.

The fusion proteins according to the present invention are composed in substance of a component corresponding to part or all of a sequence exhibiting at least 65%, preferentially at least 75%, and even more preferentially at least 85% or 95%, identity with the sequence SEQ ID No. 4 or the sequence SEQ ID No. 16, or of a substantial part of the sequence SEQ ID No. 4 or of the sequence SEQ ID No. 16, and of a component corresponding to an energy-donor or -acceptor protein. They may, however, comprise other amino acid sequences, derived from other proteins, such as signal sequences.

Advantageously, the energy-donor protein is Renilla luciferase (Rluc). It may, however, be any other energy-donor protein such that the emission spectrum of the donor overlaps the excitation spectrum of the acceptor sufficiently to allow efficient energy transfer between the two partners. It may thus be GFP, if the energy transfer is FRET, or else aequorin if the energy transfer is CRET. Aequorin can be obtained and used as described in patent application EP 0 187 519, or in the article by Inouye et al. (PNAS USA 82 : 3154-3158 (1985)).

As regards the energy-acceptor fluorescent protein, it is preferentially DsRed, GFP or a mutant of this protein, such as YFP, EYFP, wild-type GFP, GFPS65T, Topaz or GFP_{10}.
It may however be any other energy-acceptor fluorescent protein such that the excitation spectrum of the acceptor and the emission spectrum of the donor overlap sufficiently to allow efficient energy transfer between the two partners.

These proteins are known to those skilled in the art, who can find their sequences in the literature, in particular in the review by Blinks et al. (Pharmacol. Rev. 28 : 1-93 (1976)). In particular, GFP is described by Tsien (Annu. Rev. Biochem. 67 : 509-544 (1998)) and the cloning thereof is described by Prasher et al. (Gene 111 : 229-233 (1992)). As regards the cloning of DsRed, it is described by Matz et al. (Nat. Biotechnol. 17 : 969-973 (1999)). For Rluc, those skilled in the art can refer to Blinks et al. (Pharmacol. Rev. 28 : 1-93 (1976)) or else to Lorenz et al. (PNAS 88: 4438-4442 (1991)).

Particularly advantageously, the donor and acceptor fusion proteins have one of the sequences SEQ ID No. 6, SEQ ID No. 8, SEQ ID No. 12, SEQ ID No. 14, SEQ ID No. 18 or SEQ ID No. 20, or a variant of this sequence exhibiting at least 65% identity.

Other subjects of the present invention are nucleic acids encoding these proteins. Such nucleic acids may be complementary or genomic DNAs, or RNAs. These nucleic acids or polynucleotides can be in single-chain form or in the form of duplex.

They are particularly advantageously complementary DNAs.

Preferentially, a subject of the invention is a nucleic acid having at least 65%, preferentially at least 75%, and even more preferentially at least 85% or 95%, nucleotide identity with a nucleic acid of sequence SEQ ID No. 5, SEQ ID No. 7, SEQ ID No. 11, SEQ ID No. 13, SEQ ID No. 17 or SEQ ID No. 19.

According to yet another aspect, the invention relates to a nucleic acid which hybridizes, under high stringency hybridization conditions, with a nucleic acid as defined above, and more particularly a nucleic acid of nucleotide sequence SEQ ID No. 5, SEQ ID No. 7, SEQ ID No. 11, SEQ ID No. 13, SEQ ID No. 17 or SEQ ID No. 19, or a nucleic acid of complementary sequence.

For the purpose of the present invention, the "percentage identity" between two nucleotide or amino acid sequences can be determined by comparing two optimally aligned sequences through a window of comparison.
The part of the nucleotide sequence or polypeptide in the window of comparison, may thus comprise additions or deletions (for example gaps) compared to the reference sequence (which does not comprise these additions or these deletions) so as to obtain optimal alignment of the two sequences.
The percentage is calculated by determining the number of positions at which an identical nucleic acid base or amino acid residue is observed for the two (nucleic acid or peptide) sequences compared, in dividing the number of positions at which there is identity between the two bases or amino acid residues by the total number of positions in the window of

comparison, and then multiplying the result by 100 in order to obtain the percentage sequence identity.

The optimal alignment of the sequences for comparison can be produced on a computer using known algorithms contained in the WISCONSIN GENETICS SOFTWARE PACKAGE, GENETICS COMPUTER GROUP (GCG), 575 Science Doctor, Madison, WISCONSIN.

By way of illustration, the percentage sequence identity may be produced using the BLAST software (versions BLAST 1.4.9 of March 1996, BLAST 2.0.4 of February 1998 and BLAST 2.0.6 of September 1998), using exclusively the default parameters (S. F. Altschul et al, J. Mol. Biol. 1990 215 : 403-410, S. F Altschul et al, Nucleic Acids Res. 1997 25 : 3389-3402). Blast searches for sequences similar/homologous to a reference "request" sequence using the algorithm of Altschul et al. The request sequence and the databases used may be peptide-based or nucleic acid-based, any combination being possible.

For the purpose of the present invention, the expression "high stringency hybridization conditions" will be intended to mean the following conditions:

1 – Membrane competition and PRE HYBRIDIZATION:

-Mix : 40 µl of salmon sperm DNA (10 mg/ml)+ 40 µl of human placenta DNA (10 mg/ml)

- Denature for 5 min at 96°C, then immerse the mixture in ice.
- Remove the 2X SSC and pour 4 ml of formamide mix into the hybridization tube containing the membranes.
- Add the mixture of the two denatured DNAs.
- Incubate at 42°C for 5 to 6 hours, with rotation.

2- Labeled probe competition:

- Add 10 to 50 µl of Cot I DNA to the labeled and purified probe, depending on the amount of repetition.
- Denature for 7 to 10 mn at 95°C.
- Incubate at 65°C for 2 to 5 hours.

3- Hybridization :

- Remove the prehydribidization mix.
- Mix 40 µl of salmon sperm DNA + 40 µl of human placental DNA; denature for 5 min at 96°C, then immerse in ice.
- Add 4 ml of formamide mix, the mixture of the two DNAs and the denatured Cot I DNA/labeled probe to the hybridization tube.
- Incubate for 15 to 20 hours at 42°C, with rotation.

4- Washes :
- One wash at ambient temperature in 2X SSC, to rinse.
- 2 times 5 minutes at ambient temperature in 2X SSC and 0.1% SDS at 65°C.
- 2 times 15 minutes at 65°C in 1X SSC and 0.1% SDS at 65°C.
Wrap the membranes in Saran wrap and expose.

The hybridization conditions described above are suitable for hybridization, under high stringency conditions, of a nucleic acid molecule of varying length of 20 nucleotides to several hundred nucleotides.

It goes without saying that the hybridization conditions described above can be adjusted as a function of the length of the nucleic acid the hybridization of which is desired, or of the type of labeling chosen, according to the techniques well known to those skilled in the art.
The suitable hybridization conditions may, for example, be adjusted according to the teaching contained in the work by HAMES and HIGGINS (1985, "Nucleic acid hybridization : a practical approach", Hames and Higgins Ed., IRL Press, Oxford) or else in the work by F. AUSUBEL et al. (1989, Current Protocols in Molecular Biology, Green Publishing Associates and Wiley Interscience, N.Y.).

The proteins which are the subjects of the present invention can be obtained by any means known to those skilled in the art. They are, however, advantageously obtained by expression of the nucleic acids as described above, encoding these proteins, optionally inserted into expression vectors, into cells advantageously chosen, optionally followed by an extraction and a purification which may be total or partial.

The invention also relates to a recombinant vector comprising a nucleic acid according to the invention.
Advantageously, such a recombinant vector will comprise a nucleic acid chosen from the following nucleic acids:

a) a nucleic acid encoding a protein having at least 65% amino acid identity with a sequence SEQ ID No. 6, SEQ ID No. 8, SEQ ID No. 18 or SEQ ID No. 20, or a peptide fragment or a variant thereof;

b) a nucleic acid comprising a polynucleotide having a sequence SEQ ID No. 5, SEQ ID No. 7, SEQ ID NO. 17 or SEQ ID No. 19, or a fragment or a variant thereof;

c) a nucleic acid having at least 65% nucleotide identity with a nucleic acid having a sequence SEQ ID No. 5, SEQ ID No. 7, SEQ ID No. 17 or SEQ ID No. 19, or a fragment or a variant thereof;

d) a nucleic acid which hybridizes, under high stringency hybridization conditions, with a nucleic acid of sequence SEQ ID No. 5, SEQ ID No. 7, SEQ ID No. 17 or SEQ ID No. 19, or a fragment or a variant thereof.

For the purposes of the present invention, the term "vector" will be intended to mean a circular or linear DNA or RNA molecule which is indifferently in single-stranded or double-stranded form.

According to one embodiment, the expression vector comprises, besides a nucleic acid in accordance with the invention, regulatory sequences which make it possible to direct the transcription and/the translation thereof.

According to an advantageous embodiment, a recombinant vector according to the invention will in particular comprise the following elements:

(1) elements for regulating the expression of the nucleic acid to be inserted, such as promoters and enhancers;

(2) the coding sequence included in the nucleic acid in accordance with the invention to be inserted into such a vector, said coding sequence being placed in phase with the regulatory signals described in (1); and

(3) suitable transcription initiation and stop sequences.

In addition, the recombinant vectors according to the invention may include one or more origins of replication in the cellular hosts in which their amplification or their expression is desired, markers or selection markers.

By way of examples, the promoters for eukaryotic cells will comprise the thymidine kinase promoter of the HSV virus or else the mouse metallothionein-L promoter.

In general, in choosing a suitable promoter, those skilled in the art may advantageously refer to the work by SAMBROOK et al. (1989, "Molecular Cloning : A Laboratory Manual," 2nd ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.) or else to the techniques described by FULLER et al. (1996, *Immunology in Current Protocols in Molecular Biology*, Ausubel et al).

The preferred vectors according to the invention are plasmids, such as, for example, the vectors pCDNA3 (Invitrogen), pQE70, pQE60, pQE9 (Qiagen), psiX174, pBluescript SA, pNH8A, pNH16A, pNH18A, pNH46A, pWLNEO, pSV2CAT, pOG44, pXTI and pSG(Stratagene).

They may also be vectors of the *baculovirus* type, such as the vector pVL1392/1393 (Pharmingen) used to transfect cells of the Sf9 line (ATCC No. CRL 1711) derived from *Spodoptera frugiperda*.

They may also be adenoviral vectors, such as human adenovirus type 2 or 5.

A recombinant vector according to the invention may also be a retroviral vector or else an adeno-associated vector (AAV). Such adeno-associated vectors are, for example, described by FLOTTE et al. (1992, *Am. J. Respir. Cell Mol. Biol.*, **7** : 349-356).

Objects of the present invention are also cells comprising a protein, a nucleic acid or a vector as described above, or fragments of these cells, lysates of these cells or else membranes of these cells.

Such cells may be cells isolated from an organism and cultured in a suitable growth medium. They are, however, preferentially cell lines. Thus, such lines are particularly advantageously the cells lines HEK 293, COS (ATCC No. CRL 1650), COS-M6 and HeLa (ATCC No. CCL2), or else Cv 1 (ATCC No. CCL70), Sf-9 (ATCC No. CRL 1711), CHO (ATCC No. CCL-61) or 3T3 (ATCC No. CRL-6361).

The membranes of these cells can be prepared by any method known to those skilled in the art.

Preferentially, they will be prepared by mechanical grinding of the cells and then centrifugation of the suspensions obtained, as illustrated in the examples which follow.

The present invention also relates to compositions comprising cells as described above and saponin.

The present invention also relates to a method for determining the modification, by a compound, of the interaction between the OB-RGRP, the MY047 protein or a protein exhibiting at least 65% identity with the sequence SEQ ID No. 4 or the sequence SEQ ID No. 16, and the leptin receptor, comprising the steps consisting in:

- bringing said compound into contact with a protein exhibiting at least 65% identity with the sequence SEQ ID No. 4 or the sequence SEQ ID No. 16, and the leptin receptor, or cells, or fragments or lysates or membranes of cells, comprising such proteins, and optionally a suitable enzyme substrate, and

- measuring the interaction between a protein exhibiting at least 65% identity with the sequence SEQ ID No. 4 or the sequence SEQ ID No. 16, and the leptin receptor.

Preferentially, said compound is brought into contact with an energy-donor fusion protein and an energy-acceptor fusion protein, or cells, or fragments or lysates or membranes of cells, comprising such a protein, and optionally a suitable enzyme substrate.

Preferentially, said method is brought into contact with cells treated with an agent which permeabilizes these cells, such as saponin.
The energy-donor fusion proteins and the energy-acceptor fusion proteins are chosen such that the energy resulting from the activation of the donor may be transferred efficiently to the acceptor.

In an advantageous embodiment of said method, the energy-donor fusion protein is a protein from fusion with luciferase or a substantial part of luciferase, in which case the substrate is advantageously coelenterazine.

In a preferential embodiment of said method, the energy-acceptor fusion protein is a protein from fusion with YFP or a substantial part of YFP.

In an advantageous embodiment of said method, the energy transfer measured in the presence of the test compound is compared to that measured in the absence of the test compound.
In another advantageous embodiment of said method, the energy transfer measured in the presence of the test compound and of leptin (or a ligand of the receptor) is compared to that measured in the presence of the compound in the absence of leptin (or a ligand of the receptor).

Preferentially, the method is carried out on cell membranes, as described above.

Preferentially, the donor and acceptor proteins according to the present invention are chosen such that the energy transfer takes place by first or second generation BRET (for Bioluminescence Resonance Energy Transfer) or LRET (for Luminescence Resonance Energy Transfer). However, such an energy transfer may be effected by FRET (for

Fluorescence Resonance Energy Transfer) or else by CRET (for Chemioluminescence Resonance Energy Transfer).

Whatever the type of energy transfer, the energy-donor fusion protein/energy-acceptor fusion protein pairs are chosen so as to allow such transfer.

BRET2 (2nd generation) consists of energy transfer between *Renilla* luciferase and a mutant GFP, GFP_{10}, using a suitable substrate, DeepblueC™ coelanterazine (Biosignal Packard).

CRET consists of energy transfer between aequorin, which is a luciferase, and GFP.

FRET consists of energy transfer between two proteins of the GFP family having different spectra.

To implement these transfers, those skilled in the art may refer to D. Ramsay et al. (Biochem J 365: 429-40 (2002)) and to K. Yoshioka et al. (FEBS Lett 523: 147-151 (2002)) for BRET2, to Baubet et al. (PNAS USA 97 : 7260-7265 (2000)) for CRET, and to Matyus (J Photochem Photobiol B 12: 323-337 (1992)) and Pollok and Heim (Trends Cell Biol 9:57-60 (1999)) for FRET.

Another subject of the present invention is a method for screening or detecting compounds intended for the prevention and/or treatment of leptin-related pathological conditions, comprising the steps consisting in:

- bringing said compound into contact with a protein exhibiting at least 65% identity with the sequence SEQ ID No. 4 or the sequence SEQ ID No. 16, and the leptin receptor, or cells, or fragments or lysates or membranes of cells, comprising such proteins, and optionally a suitable enzyme substrate, and
- measuring the interaction between a protein exhibiting at least 65% identity with the sequence SEQ ID No. 4 or the sequence SEQ ID No. 16, and the leptin receptor.

Preferentially, the protein exhibiting at least 65% identity with the sequence SEQ ID No. 4 or the sequence SEQ ID No. 16 is the OB-RGRP or MY047.

The method according to the present invention is compatible with the 96-well or 384-well plates generally used. It does not require the use of radioactive molecules, but is sensitive, reproducible and rapid, and the result is easy to read. This characteristic is particularly advantageous for carrying out large scale screening.

The present invention also relates to the use of compounds selected using a method consisting in:
-bringing said compound into contact with a protein exhibiting at least 65% identity with the sequence SEQ ID No. 4 or the sequence SEQ ID No. 16, and the leptin receptor, or cells, or fragments or lysates or membranes of cells, comprising such proteins, and optionally a suitable enzyme substrate, and
- measuring the interaction between a protein exhibiting at least 65% identity with the sequence SEQ ID No. 4 or the sequence SEQ ID No. 16, and the leptin receptor.

A subject of the present invention is, finally, a method of curative or preventive treatment of leptin-related diseases or diseases related to its receptor, comprising the steps of:
- selecting said compound using a method consisting in:
+bringing said compound into contact with a protein exhibiting at least 65% identity with the sequence SEQ ID No. 4 or the sequence SEQ ID No. 16, and the leptin receptor, or cells, or fragments, or lysates or membranes of cells, comprising such proteins, and optionally a suitable enzyme substrate, and
+measuring the interaction between a protein exhibiting at least 65% identity with the sequence SEQ ID No. 4 or the sequence SEQ ID No. 16, and the leptin receptor
- administering said compound to a patient suffering from said disease.

Leptin-related pathological conditions may be diseases related to a decrease in bone density, such as, for example, osteoporosis, or, conversely, those related to considerable calcification.
They may also be diseases which have an effect on weight, such as obesity, diabetes or anorexia.
They may also be diseases which have an effect on sexual maturation, hematopoiesis, angiogenesis, thrombus formation, the regulation of immunity and inflammation, fetal development, cicatrization and cancer.

The compounds of the invention, oligonucleotides, iRNAs, or other compounds, may be formulated in pharmaceutical compositions for the purpose of topical, oral, parenteral, intranasal, intravenous, intramuscular, subcutaneous, intraocular administration, etc. Preferentially, the pharmaceutical compositions contain pharmaceutically acceptable vehicles

for an injectable formulation. They may in particular be isotonic, sterile, saline (monosodium or disodium phosphate, sodium, potassium, calcium or magnesium chloride, etc., or mixtures of such salts) solutions, or dry, in particular lyophilized, compositions which, by addition, as appropriate, of sterilized water or of physiological saline, make it possible to constitute injectable solutes.

The formulation of therapeutic compositions and their administration fall within the competence of those skilled in the art.

The formulation of the compounds may include various products known to those skilled in the art. Preferentially, the compounds may, for example, have salts, such as sodium, potassium, ammonium, magnesium, calcium, polyamines, or hydrochloric, hydrobromic, sulfuric, phosphoric or nitric acid, added to them. Other salts can also be used, such as those originating from acetic, oxalic, tartaric, succinic, maleic, fumaric, gluconic, citric, malic, ascorbic, benzoic, tannic, palmitic, alginic, polyglutamic, naphthalene-sulfonic, methanesulfonic, p-toluenesulfonic, naphthalenedisulfonic or polygalacturonic acid. Finally, chlorine, bromine and iodine salts can also preferentially be used.

The composition and the formulation for topical administration can include transdermal patches, ointments, lotions, creams, gels, drops, suppositories, sprays, liquids and powders.

The composition and the formulation for oral administration can include powders, granules, microparticles, nanoparticles, suspensions, solutions, which may or may not be aqueous, capsules, gelatin capsules, sachets, tablets or mini tablets. Thickeners, flavors, diluents, emulsifiers, dispersing agents or binders may be added.

The composition and the formulation for parenteral, intrathecal or intravententricular administration can include sterile aqueous solutions which can also contain buffers, diluents and other additives, such as, but not limited to, penetration-increasing agents, transporting products and excipients.

The composition can be formulated and used as a foam, an emulsion, a microemulsion, cationic, pH-sensitive or negatively charged liposomes, and transferomes.

In general, the various formulations can contain a mixture of one or more agents, such as, but not limited to, agents which increase the penetration of the compound (surfactants, bile salts, chelating agents, non-chelating surfactants), excipients (binders, fillers, lubricants, disintegrating agents, wetting agents), or transporters (water, saline solutions, alcohols, polyethylene glycol, gelatin, lactose, amylose, magnesium stearate, talc, silicic acid, viscous paraffin, hydroxymethylcellulose, polyvinylpyrrolidone). Other components can be added, such as dyes, flavors, preserving agents, antioxidants, opacifiers, thickeners and stabilizers.

The dosage depends on the severity and on the sensitivity of the state of the disease to be treated, with a treatment period possibly ranging from a few days to a few months, or until the treatment is effective or a reduction in the disease is observed. The optimum dosage can be calculated from measurements of accumulation of the therapeutic agent in the patient's body. Those skilled in the art can easily determine the optimum dosages, the methods of dosage and the rates of repetition of these dosages. The optimum dosages can vary as a function of the relative effectiveness of each oligonucleotide or iRNA, and can, in general, be estimated by measuring the EC50s of the doses used in vitro and in vivo in animal models. In general, the dosage is between 0.01µg and 100 g per kilo of bodyweight and can be administered one or more times, daily, weekly, monthly or annually, or even once every 2 to 20 years.
Competent individuals can easily determine the rate of repetition of the dosages based on the amount of time the compound is present in the body fluids or the tissues. Subsequent to a successful treatment, it may be desirable for the patient to continue a maintenance therapy in order to prevent reappearance of the disease; to do this, the oligonucleotide or the iRNA is administered at maintenance doses ranging from 0.01 µg to 100 g per kilo of bodyweight, one or more times a day, up to once every 20 years.

The administration of antisense in vivo has been carried out successfully by various authors, using protocols of simple injection of antisense intravenously (He et al. (1998) Zhonghua Shi Yan He Lin Chuang Bing Du Xue Za Zhi 12:1-4) or intracerebrally (Yoburn et al. (2003) Synapse 47: 109-116, Tischkau et al. (2003) J. Biol. Chem. 278: 718-723). In the last two years, more complex systems for targeting antisense in the organism have been developed and used successfully (Morishita et al. (2002) J. Endocrinol. 175: 475-485, Bartsch et al. (2002) Pharm. Res. 19: 676-680),

making it possible, in mice and rats, to treat various cancers (Rait et al. (2002) Mol. Med. 8: 475-486, Ochietti et al. (2002) J. Drug. Target 10: 113-121, Eder et al. (2002) Cancer Gene Ther. 9:117-125). The transfection of antisenses involves the same methods as for the transfection of iRNAs, making it possible to envision the same applications in vivo for the iRNAs. With this in mind, it is possible to imagine targeting the antisense or the iRNAs to the central nervous system, in order to treat disorders of central origin (obesity), but also those produced by a peripheral action of leptin receptors. More particularly, it is possible to envision there being an action of the antisenses or of the iRNAs on the transport of leptin across the blood-brain barrier, involving OB-R. Moreover, endothelial cells have already been successfully targeted using an in vivo antisense strategy (Bartsch et al. (2002) Pharm. Res. 19: 676-680).

Figures :

Figure 1
Sequences of the various antisense ODNs used, AS 01 to AS 16.

Figure 2
Alignment of the OB-RGRP protein sequences of various species and of the human MY047 protein sequence. The potential transmembrane domains were determined by various methods (HMMTOP, TMHMM, TopPred2, TMpred) and are written in bold.

Figure 3
Topology of OB-RGRP studied by BRET, using the double fusion protein YFP-OB-RGRP-Luc. Figure 3a: diagrammatic representation of the topology of OB-RGRP for the models 3 and 4TM. Figure 3b: results of the BRET experiments using the proteins indicated. The data are expressed in mBU.

Figure 4
Study of the oligomerization of OB-RGRP with SDS-PAGE experiments and immunoprecipitations. Figure 4a: the cells expressing the fusion proteins indicated were treated or not treated with 2 $mmol.L^{-1}$ dithiobis(succinimidyl propionate) (DSP) in PBS (1X, pH7.4) in order to crosslink the protein complexes. The proteins were separated by SDS-PAGE and the proteins from fusions with YFP were detected using a

specific anti-YFP antibody. Figure 4b: the cells expressing the construct 6Myc-OB-RGRP were solubilized with 1% of digitonin or 5% of SDS and the solubilized material was immunoprecipitated with an anti-myc antibody. The precipitates were subjected to separation by SDS-PAGE and the proteins tagged with myc were detected with an anti-myc antibody.

Figure 5

Identification of the molecular determinants involved in the oligomerization of OB-RGRP. The proteins from fusions with the OB-RGRP truncations were treated as described in Fig. 4b. TM, transmembrane domain.

Figure 6

Study of the oligomerization of OB-RGRP in live HEK cells, by BRET technology. Figure 6a: the fusion proteins indicated were coexpressed at an equimolar ratio, and BRET measurement experiments were carried out. Figure 6b: constant amounts of the plasmid OB-RGRP-Luc were coexpressed with increasing amounts of the plasmid OB-RGRP-YFP and BRET measurements were carried out. MT2R-Luc, protein from fusion of the melatonin receptor MT2 with luciferase.

Figure 7

Interaction of OB-R_s and of OB-RGRP studied by BRET. The fusion proteins indicated were coexpressed at an equimolar ratio and BRET measurements were carried out. IR-YFP, protein from fusion of the insulin receptor with YFP.

Figure 8

Dose-dependent activation of the reporter genes for STAT3 (Figure 8a) and STAT5 (Figure 8b) in HeLa cells, by OB-R_l in the presence of overexpression of the OB-RGRP protein constructs as indicated.

Figure 9

Effect of the overexpression of OB-RGRP on the expression of OB-R at the surface of the cells. HEK 293 cells transfected or not transfected with the OB-RGRP expression vector, and COS cells transfected with the OB-R_l or OB-R_s expression vectors and +/- the OB-RGRP vectors, were used to determine the amount of receptors expressed at the surface and the total expressed in the cells, by ^{125}I-leptin-binding experiments.

Figure 10

Effect of the various antisense oligodeoxynucleotides (ODNs) on the level of OB-RGRP messengers observed by semiquantitative RT-PCR. Figure 10a: determination of the linear zone of amplification of the OB-RGRP and GAPDH transcripts, as a function of the number of PCR cycles. Figure 10b: quantification of the results shown in panel a. Figure 10c: determination of the relative levels of expression of the OB-RGRP mRNAs at 26 PCR cycles, in the cells incubated with the various antisense ODNs.

Figure 11

Effect of the various interfering RNAs on the level of OB-RGRP messengers observed by semi-quantitative RT-PCR. **Figure 11a**: sequence SEQ ID No. 37/SEQ ID No. 38 of the synthetic iRNA used (homolog for humans and mice). **Figure 11b**: determination of the relative expression levels of the OB-RGRP mRNAs at 26 PCR cycles, in untransfected HELA cells or HELA cells transfected with the synthetic iRNA. **Figure 11c**: sequence SEQ ID No. 42 of the hairpin iRNA synthesized from the vector PCR3.1-RNAi 14. **Figure 11d**: determination of the relative expression levels of the OB-RGRP mRNAs at 26 PCR cycles, in untransfected Ltk cells or Ltk cells transfected with the vector PCR3.1-RNAi 14.

Figure 12

Effect of the OB-RGRP-specific antisense ODNs on the activation of a STAT3 reporter gene. The HeLa cells were cotransfected firstly with the OB-RI expression vector and the constructs of the reporter genes for STAT3 or 5, and then with the antisense ODNs indicated. After 48 hours of stimulation or no stimulation with 10 $nmol.L^{-1}$ of leptin.

Figure 13

Effect of the OB-RGRP-specific antisense ODNs on the surface expression of OB-R. The HeLa cells were transfected or not transfected with the $OB\text{-}R_l$ or $OB\text{-}R_s$ expression plasmids, before a second transfection with the antisense ODNs indicated, or no second transfection. 48 h post-transfection, the total amount of OB-R and the fraction exposed at the surface were determined in binding experiments with ^{125}I-leptin.

The present invention is illustrated, without, however, being limited, by the following examples.

Materials and methods used in the examples

Plasmid construction

The proteins from fusion of OB-R with YFP and luciferase were constructed by ligation of YFP and of luciferase to the C-terminal portion of the OB-R receptors, by standard molecular biology techniques. The coding region of YFP was obtained from the vector Cytogem®-Topaze (pGFPtpz-N1) (Packard, Meriden, CT) and was inserted into the EcoRV site of the vector pcDNA3/CMV (Invitrogen, Groningen, The Netherlands) containing a modified polylinker. The coding region of *Renilla* luciferase was obtained from the vector pRL-CMV (Promega, Madison, WI) and inserted into the EcoRV site of the modified vector pcDNA3. The coding regions of OB-R_l and of OB-R_s (a gift from Dr. Gainsford, Royal Melbourne Hospital, Victoria, Australia) were inserted into the two vectors described above, respectively into the EcoR1/BamH1 and Nhe1 sites. The stop codons were deleted by site-directed mutagenesis and the frame of the fusion proteins was adjusted at the same time.

The vector pcDNA3-OB-RGRP was obtained by insertion of the coding region of OB-RGRP, obtained from the vector pCDNA3-Di1, into the EcoR1 and Xba1 sites of the vector pcDNA3/CMV (Invitrogen, Groningen, The Netherlands). The stop codon of OB-RGRP was deleted by site-directed mutagenesis. The vector pcDNA3-OB-RGRP-Luc was obtained by digestion of the vector pRL-CMV N3 (Promega, Madison, WI) with Sma1 and Hpa1 and by insertion of the fragment corresponding to the coding region of *Renilla* luciferase, after the coding region of OB-RGRP, into the filled-in BspE1 site of the vector pcDNA3-OB-RGRP.

The vector pcDNA3-YFP was obtained by subcloning the coding region of YFP from the vector pGFPtpz-N1 (Packard, Meriden, CT) inserted into the EcoRV site of the vector pcDNA3/CMV. The vector pcDNA3-OB-RGRP-YFP was obtained by insertion of the BamH1/BspE1 fragment of the vector pCDNA3-OB-RGRP non-stop into the vector pcDNA3-YFP digested with the BamH1 and Age1 enzymes.

The construct pcDNA3-GFP-OB-RGRP-Luc was obtained by insertion of the OB-RGRP-Luc fragment of the vector pcDNA3-OB-RGRP-Rluc, cleaved with EcoR1, into the EcoR1 site of the vector pcDNA3-YFP. The stop codon of the YFP was removed by site-directed mutagenesis.

The vector 6Myc-OBR-GRP (4TM) was obtained by insertion of the 6myc fragment of the vector pCDNA3-RSV-6Myc into the BamH1 and EcoR1

sites of the vector pCDNA3-OBRGRP. The various OB-RGRP deletions (2 and 3 TM) were obtained by PCR and the insertion into the vector pcDNA3, into the EcoR1 and Xba1 sites. The coding sequence of MY047 was obtained by RT-PCR on mRNAs of human origin. The PCR fragment was digested with the EcoR1/Xba1 restriction enzymes and inserted into the vector pcDNA3-Topaze cleaved with the same enzymes. The stop codon of the YFP was then removed by site-directed mutagenesis, so as to obtain the vector pcDNA3-YFP-MY047. The vector pcDNA3-MY047-YFP was obtained by insertion of the DNA fragment obtained by PCR on the vector pcDNA3-YFP-MY047 and cleaved with BamH1, then inserted into the vector pcDNA3-YFP cleaved with the same enzyme. Insertion of the same fragment into the vector pcDNA3-Rluc cleaved with BamH1 made it possible to obtain the vector pcDNA3-MY047-Rluc. Two consecutive PCR reactions were carried out so as to obtain amplification of the mouse U6 promoter followed by the hairpin iRNA sequence of sequence SEQ ID No. 42. In a first reaction, a first pair of primers: U6 sense, 5′-CCATCTAGGCCAAGCTTATCCGACGCCGCCATCTC-3′
SEQ ID No. 41 and that corresponding to the sense sequence of the target followed by a sequence forming the loop (SEQ ID No. 39). This PCR product was then used in a second reaction with the same sense primer (U6 sense) and a second primer corresponding to the antisense sequence of the target preceded by the same sequence forming the loop (SEQ ID No. 40). The various PCR products corresponding to the various hairpin iRNAs were inserted into the vector PCR3.1 using the TA-cloning kit (INVITROGEN, Groningen, the Netherlands), to give the plasmids PCR3.1-RNAi 14. All the constructs were verified by sequencing.

Cell culture and transfection

The HEK 293, COS-7 and HeLa cells were cultured in DMEM supplemented with 10% (v/v) of SVF, 4.5 g/liter of glucose, 100 U/ml of penicillin, 0.1 mg/ml of streptomycin and 1 mmol.L^{-1} of glutamine (all from Life Technologies, Gaithersburg, MD). The transient transfections were carried out with the FuGene 6 reagent (Roche, Basle, Switzerland) according to the supplier's instructions, except for the Ltk cells. The latter were transfected using the DEAE dextran technique: the cells are rinsed twice with PBS, and then 1 ml of a mixture of 2 μg of DNA, DMEM, 20 mM Hepes, 4.5 g/liter of glucose and 200 μg of DEAE-dextran is added to the cells. After incubation for 8 hours, the medium is removed and the cells are then incubated for 1 hour 30 min with 1 ml of DMEM, 4.5 g/liter of glucose

and 10% DMSO. The cells are finally rinsed and then incubated with the culture medium.

Preparation of membranes and solubilization

The membranes were prepared as previously described (19), and resuspended in 75 mmol.L^{-1} Tris (pH 7.4), 12.5 mmol.L^{-1} $MgCl_2$ and 5 mmol.L^{-1} EDTA, and immediately used in BRET experiments.

SDS PAGE and Western blotting

The total lysates were prepared by washing the cells once with cold PBS (pH 7.4) and denatured by adding loading buffer (30 mmol.L^{-1} Tris HCl, pH 6.8, 1% glycerol, 5% SDS, 50 mmol.L^{-1} DTT and 0.05% bromophenol blue). The total lysates or the immunoprecipitates were incubated for 10 minutes at 90°C and then loaded onto 10% acrylamide gel for separation by electrophoresis (SDS-PAGE). The proteins were then transferred onto a nitrocellulose membrane and revealed with specific primary antibodies: anti-YFP (8367-1 Living Colors) diluted to 1/200, anti-myc A14 (*sc-789* TEBU Peprotech Santa Cruz Biotechnology) diluted to 1/500, then a secondary antibody coupled to peroxidase (anti-rabid goat IgG; Jackson Immunoresearch Laboratories, Inc., West Baltimore Pike) diluted to 1/10,000. The immunoreactive bands were revealed with an ECL kit (Pharmacia Biotech).

Immunoprecipitation

Two days after transfection, the cells were washed once with cold PBS, and the proteins were extracted by incubation for 15 minutes in lysis buffer (1X PBS, 1% Nonidet P40, 0.5% sodium deoxycholate, 0.1% SDS, 0.02% NaN_3, 10 mg.L^{-1} benzamidine and 5 mg/L^{-1} trypsin inhibitors). The lysate was centrifuged at 18000 g for 15 min and the supernatant was then incubated for 3 hours at 4°C with an anti-myc antibody coupled to agarose beads (*sc 40AC* TEBU preprotech, Santa CRUZ Biotechnology). The precipitates were washed three times with cold lysis buffer and denatured with loading buffer for SDS-PAGE.

Radiobinding experiments

The radiobinding experiments were carried out as previously described (Barr et al., (1999) J Biol Chem, 274, 21416-21424), with slight modifications. To determine the surface leptin binding, the cells cultured in the 6-well plates were washed twice with cold PBS and incubated in the

binding buffer (DMEM, 25 mmol.L^{-1} Hepes, pH 7.4, 1% BSA) containing 100,000 cpm/well of ^{125}I-leptin (PerkinElmer life sciences, Paris, France) in the presence or absence of 200 nmol.L^{-1} of leptin (PreproTech Inc, USA) for 4 h at 4°C. The cells were washed twice with cold PBS, then lyzed in 1N NaOH, and the radioactivity was determined in a γ counter. To determine the total binding of leptin, the cells cultured in dishes 10 cm in diameter were solubilized in 1.5 ml of binding buffer containing 0.15% of digitonin, for 2 h at 4°C. The extracts were centrifuged for 30 min at maximum speed and at 4°C. The supernatants (0.2 ml) were incubated with 100,000 cpm of ^{125}I-leptin in the presence or absence of 200 nmol.L^{-1} of leptin, in a total volume of 0.25 ml, with constant rotation at 4°C overnight. 0.5 ml of γ-globulin (1.25 mg/ml) and 0.5 ml of polyethylene glycol 6000 (25% w/v) were added in order to precipitate the receptor-ligand complexes, which were centrifuged at 17,000 g for 3 min. The pellet was washed once with 1 ml of polyethlyene glycol 6000 (12% w/v) and the radioactivity was determined in a γ-counter.

Reporter gene activation assay

The HeLa cells cultured in wells of 6-well plates were cotransfected with 500 ng of a reporter plasmid expressing *firefly* luciferase under the control of STAT3 or STAT5 factor response elements (a gift from Dr. Levy, University of New York, New York, USA), 250 pg of the expression vector pcDNA3-*Renilla* luciferase (used as internal standard between the samples) and with 500 ng of the various OB-R expression vectors or the vector alone. 48 h after transfection, the cells were starved overnight in Optimem medium (Invitrogen, Groningen, The Netherlands) containing 1% of BSA, before stimulation with 10 nmol.L^{-1} of leptin, or no stimulation, for 48 h. The cells were then washed once with PBS, then lyzed in passive lysis buffer (Promega Corporation, Madison, WI) for 15 min at ambient temperature. The total lysates were centrifuged for 2 min at 15,000 g and the supernatants were used in an assay to measure luciferase (Dual Luciferase Assay System from Promega Corporation, Madison, WI) using a Berthold luminometer (Lumat LB 9507). The results are expressed as ratio of *firefly* luciferase activity to *Renilla* luciferase activity.

BRET measurements in microplates

48 h after transfection, the COS-7, HeLa or HEK 293 cells expressing the OB-R fusion proteins were detached and washed in PBS. 1-2x10^5 cells were distributed into wells of optiplate plates (96-well, Packard Instrument

Company, Meriden, CT) in the presence or absence of the ligands, and incubated at 25°C. Alternatively, the same procedure was carried out with membranes prepared from the cells expressing the various constructs. The substrate, coelenterazine h (Molecular Probes, Eugene, OR), was added at a final concentration of 5 µmol.L^{-1} and the readings were carried out with a Fusion™ luminometer/fluorimeter (Packard Instrument Company, Meriden, CT), which makes it possible to measure luminescence through two filters (luciferase filter: 485 ± 10 nm; YFP filter: 530 ± 12.5 nm). The BRET ratio was defined as the difference in emission at 530 nm/485 nm of the cells cotransfected with the Luc and YFP fusion proteins and the emission at 530 nm/485 nm of the Luc fusion protein transfected alone into the cells. The results are expressed as milliBRET units (mBU), 1 mBRET corresponding to the values of the differences in the ratios multiplied by 1000.

RT-PCR

The total RNAs were extracted by the method of Chomczynski and Sacchi (Chomcynzki P., and Sacchi N. (1987) Anal. Biochem. 162, 156-159). 1 µg of RNA is denatured for 5 minutes at 68°C and then abruptly cooled for 5 min at 4°C. The denatured sample is reversed transcribed for 1 h at 37°C in 20 µl of RT reaction medium (5 µmol.L^{-1} PdN6, 10 µmol.L^{-1} DTT, 50 mmol.L^{-1} Tris-HCl, pH=8.3, 75 mmol.L^{-1} KCl, 5 mmol.L^{-1} $MgCl_2$, 500 µmol.L^{-1} dNTP, 200U RT-MMLV). A 2.5 µl aliquot of this reaction is used for a PCR reaction in a final volume of 25 µl (40 mmol.L^{-1} Tris-HCl, pH 8.4: 100 mmol.L^{-1} KCl; 1.5 mmol.L^{-1} $MgCl_2$; 0.2mmol.L^{-1} of each dNTP; 0.141 mmol.L^{-1} of primers specific for OB-RGRP (human sense: CCGTGGCAGGAAGC **SEQ ID No. 43**, murine sense: GCAGCCACAGCCCCAGCTCC **SEQ ID No. 44**, antisense: CAGCCACACGAGCAAG **SEQ ID No. 45**) and 0.035 mmol.L^{-1} of primers specific for glyceraldehyde phosphate dehydrogenase (GAPDH) (sense: GGAGAAGGCTGGGGC **SEQ ID No. 46**, antisense: GATGGCATGGACTGTGG **SEQ ID No. 47**) and 2.5U of TAQ DNA polymerase). The following protocol was used for the PCR reaction: Initial denaturation for 3 min at 94°C, then 22 to 30 cycles of denaturation (20 sec at 94°C), hybridization (20 sec at 59°C), elgonation (20 sec at 72°C) followed by a final elongation of 7 min at 72°C.

An aliquot of the PCR reaction was loaded onto a 2% agarose gel in order to separate the reaction products by electrophoresis. The expected sizes of

fragments of GAPDH and of OBR-GRP are, respectively, 229 bp and 334 bp.

Oligonucleotide synthesis

The oligonucleotides were synthesized on an automatic DNA synthesizer ("Expedite MOSS" 8909 model from Applied Biosystems) by standard phosphoramidite chemistry and iodine oxidation. The demethylation was carried out with a 0.2 $mol.L^{-1}$ solution of 3H-1,2-benzodithiol-3-one 1,1-dioxide in acetonitrile for 120 s. The detachment from the support and the deprotection were carried out in concentrated ammonia (18 h at 55°C), and the oligonucleotides were then purified by precipitation. The deprotection product was precipitated with 10 volumes of 1-butanol; the pellet taken up in one volume of 0.3 $mol.L^{-1}$ NaCl was reprecipitated by adding 4 volumes of ethanol.

The analysis on a 20% polyacrylamide gel (in a buffer of 8 $mol.L^{-1}$ urea and 454 $mmol.L^{-1}$ Tris-borate, at pH 7.0) showed a greater than 80% proportion of product of expected length.

Transfection of the synthetic antisense oligodeoxynucleotides and interfering RNA duplex

For the transfection of 300,000 cells cultured in a well of a 6-well plate, 10 µl of antisense ODN at 20 $\mu mol.L^{-1}$ or 10 µl of the interfering RNA duplex at 20 µM were diluted in 175 µl of DMEM. 3 µl of oligofectamine (Invitrogen, Groningen, The Netherlands) and 12 µl of DMEM were incubated in a second tube for 10 min at ambient temperature. The oligofectamine/DMEM mixture was then added to the diluted antisense ODN, vortexed and incubated for 20 min at ambient temperature. During this time, the cells were washed once with PBS and once with DMEM, and then covered with 800 µl of DMEM. The ODN/oligofectamine mixture was then added dropwise to the cells and incubated for 4 h at 37°C, before adding 500 µl of DMEM supplemented with 30% serum.

Example 1 : Topology and cellular location of OB-RGRP

To study the topology and the subcellular location of OB-RGRP, the protein was tagged with the yellow variant of green fluorescent protein (YFP) at the end of its C-terminal tail. The fusion protein was expressed in HeLa cells and its location was determined by fluorescence microscopy.

The results show that the fusion protein is preferentially targeted to the perinuclear membranes and into intracellular vesicles. Similar results were observed in HEK cells. No colocalization with cytoplasmic and nuclear proteins was observed, confirming the location of OB-RGRP in membranes (not shown). The exact nature of the membrane compartment was determined by colocalization studies with markers specific for subcellular compartments. A strong colocalization was observed with the invariant chain of MHC II molecules, a marker for the endocytic compartment.

Initial analysis of the topology of OB-RGRP suggested an organization in 3 transmembrane (TM) domains (Bailleul et al. (1997) Nucleic Acids Research 25, 2752-2758). A similar organization has been proposed for MY047 (Huang et al. (2001) Biochimica et Biophysica acta. Gene structure and expression 327-331). However, a new analysis of the hydrophobicity profile of the various protein sequences available for OB-RGRP and MY047 is also compatible with a 4-TM model (Fig. 2). The topology differs profoundly between these two models. In the 3-TM model, the N- and C-terminal ends are located on each side of the membrane, whereas in the 4-TM model, the two tails are oriented on the same side of the membrane (Fig. 3a). To determine the correct model, we used the resonance energy transfer (BRET) method which has recently been developed to follow protein-protein interactions in living cells (Xu et al. (1999) Proc Natl Acad Sci USA 96, 151-156). In the event of physical proximity (< 100 Å between the two interacting proteins, an energy transfer can take place between the energy-donor (Luc) and the energy-acceptor (YFP), fused to the two proteins of interest. We tagged the N-terminal tail of OB-RGRP with YFP, and the C-terminal tail with luciferase, and we observed the energy transfer by measuring BRET with this double fusion protein. The 3-TM model does not allow transfer since the two BRET partners are separated by the lipid bilayer. On the other hand, the 4-TM model predicts strong energy transfers since the two partners are located on the same side of the membrane. As shown in Figure 3b, a very strong energy transfer was detected for the double fusion protein in the intact cells, indicating that OB-RGRP has 4-TMs.

This set of results suggests that OB-RGRP is a membrane-bound protein with 4 transmembrane domains, having 3 short loops and short N- and C-terminal ends oriented on the same side of the membrane. OB-RGRP is mainly located in intracellular compartments.

Example 2: Oligomerization of OB-RGRP

Oligomerization is a property common to various proteins, including membrane-bound proteins such as tyrosine kinase receptors, cytokine receptors and phosphotyrisine phosphatases. It has been shown that this oligomerization plays an important role in the function of these proteins. To obtain elements in the function of OB-RGRP, we wanted to know whether this protein oligomerizes.

OB-RGRP was tagged with YFP at its C-terminal tail and expressed in HeLa cells. The proteins were separated by polyacrylamide gel electrophoresis under denaturing conditions (SDS-PAGE) and immunoblotting experiments were carried out with an anti-YFP antibody. Figure 4a reveals several bands specific for OB-RGRP-YFP, corresponding to monomeric and dimeric forms and oligomeric complexes. Similar results were obtained with OB-RGRP tagged at the N-terminal, either with YFP or a myc epitope (Fig. 4 a,b). Formation of the OB-RGRP oligomers was observed on total cell extracts after immunoprecipitation. The use of a crosslinker on whole cells stabilizes the dimeric complexes, indicating that the dimeric form is the predominant form of OB-RGRP in intact cells (Fig. 4a).

Surprisingly, OB-RGRP has unexpected properties since the oligomers are stable in the presence of various denaturing and/or dissociating agents such as 5% SDS, 1% Triton X-100, 1% Nonidet P40, 1% digitonin, 50 mmol.L^{-1} DTT and 2% β-mercaptoethanol. However, similar observations were obtained for other membrane-bound proteins such as glycophorin A and G protein-coupled β2-adrenergic receptors. Studies on these proteins show, respectively, that **L**IXX**G**VXX**G** and **L**XXX**G**XXX**G**XXX**L** motifs in the transmembrane domains are essential for oligomer formation. Similar motifs were identified in the membrane regions of OB-RGRP.

To identify the molecular determinants involved in the dimerization, we prepared OB-RGRP constructs exhibiting progressive deletions of the C-terminal tail (Fig. 5). A construct containing the first two potential TMs loses the ability to form oligomers. Addition of the 3rd TM restores the possibility of forming dimers. However, the complete oligomerization profile was only observed in the presence of the 4 potential TMs.

Oligomers of membrane-bound proteins can be artifacts induced during the preparation of samples (solubilization, denaturation, etc.). For this reason, it is important to verify the oligomerization of proteins in living cells. Recently developed energy transfer techniques such as BRET make it possible to

follow such protein-protein interactions in living cells. Fusion proteins of OB-RGRP with luciferase and YFP were used to follow OB-RGRP oligomerization in living cells. Coexpression of the OB-RGRP-YFP or YFP-OB-RGRP constructs with the OB-RGRP-Luc construct induces an energy transfer (Fig. 6a). The specificity of this interaction was shown by the lack of energy transfer during the coexpression with two different fusion proteins: β-arrestine2-YFP (Angers et al. (2000) Proc Natl Acad Sci USA 97, 3684-3689), or melatonin-Luc MT2 receptor (Ayoub et al. (2002) J Biol Chem 277, 21522-21528). We then expressed various ratios of the BRET partners (Fig. 6b). The BRET signal is increased in a hyperbolic manner as a function of the OB-RGRP-YFB/OB-RGRP-Luc ratio, reaching an asymptote which corresponds to saturation of the energy-donor molecules (OB-RGRP-Luc) by the acceptor molecules (OB-RGRP-YFP), which is expected in the case of a specific interaction.

Collectively, these results show that OB-RGRP is a dimeric membrane-bound protein which can also be involved in high molecular weight oligomeric complexes. The 3rd and 4th potential transmembrane domains appear to be important for oligomer formation.

Example 3: Interaction between OB-R and OB-RGRP and MY047

We used BRET technology to study a possible interaction between OB-R and OB-RGRP in living cells. An energy transfer was constitutively observed in the cells coexpressing the OB-R_s-Luc construct and the OB-RGRP-YFP construct, indicating proximity of the interaction partners (Fig. 7). The same results were obtained in cells coexpressing OB-R_s-Luc and the MYO47-YFP construct, and also in the reverse orientation: in cells coexpressing OB-RGRP-Luc and OB-R_s-YFP, or in cells coexpressing MYO47-Luc and OB-R_s-YFP. The specificity of these interactions was confirmed by the lack of energy transfer between OB-R_s-Luc, OB-RGRP-Luc, MYO47-Luc and a construct of the insulin receptor tagged with YFP (Boute et al. (2001) Mol Pharmacol 60, 640-645), and also in the reverse orientation: by the lack of energy transfer between a construct of the insulin receptor tagged with Luc and the OB-R_s-YFP, OB-RGRP-YFP and MYO47-YFP construct. Coexpression of OB-R_s-Luc and an OB-RGRP or MYO47 construct exhibiting the YFP tag at the N-terminal produces no significant signal, confirming the specificity of interaction with OB-RGRP-YFP and MYO47, and indicates that the N-terminal end of OB-RGRP and MYO47 must be involved in the interaction with OB-R.

No significant energy transfer was observed in the cells coexpressing the OB-R_l-Luc and OB-RGRP-YFP or YFP-OB-RGRP constructs. This is not due to a lack of functional OB-R_l-Luc expression since a specific BRET signal was observed in cells coexpressing OB-R_l-YFP in order to follow OB-R dimerization. The lack of BRET between the OB-R_l-Luc and OB-RGRP-YFP fusion proteins does not exclude a direct interaction between these two proteins since this may be explained by the fact that the distance between the two BRET partners (Luc and YFP) is greater than 100 Å, the maximum distance for obtaining a transfer. This should be the case since the N- and C-terminal ends of OB-RGRP should be located close to the transmembrane region of OB-R, whereas the C-terminal end of OB-R_l should more probably point toward the cytoplasm due to its long intracellular tail of approximately 300 amino acids. Given that the short and long isoforms of OB-R share the same trans- and juxtamembrane regions and that the interaction of OB-RGRP with OB-R_s is located at this level, it is probable that OB-RGRP interacts with OB-R_l in the same way as with OB-R_s.

Example 4: Effect of the overexpression of OB-RGRP on OB-R signaling

Constructs containing STAT3- or STAT5-response elements upstream of a luciferase reporter gene were coexpressed with OB-R_l in the presence or absence of various OB-RGRP constructs (Fig. 8). The two constructs were activated by leptin in a dose-dependent manner, with an EC50 of approximately 50 pM. Similar results were obtained in HEK 293 cells stably expressing a reporter gene for STAT3. The overexpression of various OB-RGRP constructs had no reproducible effect on this activation, indicating that OB-RGRP is not a limiting factor.

Example 5: Effect of the overexpression of OB-RGRP on the expression of OB-R at the surface

In yeast knockout for OB-RGRP (Vps55), protein transport is disturbed between the golgi and the vacuoles (Belgareh-Touze et al. (2002) Molecular Biology Of The Cell 13, 1694-1708). Although OB-R are activated only when they are expressed at the plasma membrane, a considerable amount of receptors is accumulated in intracellular compartments (Barr, et al. (1999) J Biol Chem, 274, 21416-21424) (Lundin

et al. (2000) Biochimica and Biophysica Acta 1499, 130-138). For this reason, we tested the effects of the overexpression of OB-RGRP on the expression of OB-R at the cell surface.

The receptor distribution was studied by ^{125}I-leptin-binding experiments. In agreement with other authors (Barr et al., 1999), we showed that only 10 – 20% of the OB-R_l and OB-R_s receptors are expressed at the surface of transfected COS cells (Fig. 9) and of HeLa cells. This is not an artifact due to the expression of exogenous receptors since similar values are obtained in HEK 293 cells expressing endogenous OB-R receptors (Fig. 9). The overexpression of OB-RGRP showed no modification of the total amount in the cells, nor of the % of receptors expressed at the surface (Fig. 9).

Example 6: Characterization of OB-RGRP specific antisense deoxynucleotides

OB-RGRP appears to have ubiquitous expression; for this reason, the decrease in expression of this protein was chosen as an alternative approach for studying its role in OB-R function. Fourteen antisenses specific for OB-RGRP (AS 1 to 14; **SEQ ID No. 22 to SEQ ID No. 34 and SEQ ID No. 2**) and two random antisenses (AS 15 and 16; **SEQ ID No. 35 and SEQ ID No. 36**) were chosen (see Figure 1), synthesized, and then tested for their ability to inhibit OB-RGRP expression using semiquantitative RT-PCR experiments in HeLa cells expressing OB-RGRP endogenously (Fig. 10). Only one of these antisenses (AS-14), derived from the untranslated 3' region of the OB-RGRP mRNA, interferes with OB-RGRP expression. Labeling of this antisense with the Cy3 fluorophore made it possible to show that all of the cells were transfected under our experimental conditions, in our various experiments.

Example 7: Effect of the various interfering RNAs on the level of OB-RGRP messengers observed by semi-quantitative RT-PCR

In order to decrease the expression of OB-RGRP, an alternative approach was to use interfering RNAs. For this, we used, firstly, a synthetic interfering RNA candidate sequence directed against both the human and murine sequence (figure 11a) and, secondly, a vector (PCR3.1-RNAi 14) expressing a hairpin interfering RNA directed against a murine sequence of OB-RGRP (figure 11c).

The ability of the iRNAs to decrease the endogenous OB-RGRP expression was tested by RT-PCR.

The synthetic iRNA transfected into HELA cells (of human origin) causes a decrease in the expression of human OB-RGRP.

Transfection of the vector PCR3.1-RNAi-14 causes the same effect in L cells (of murine origin).

Example 8: Effect of the OB-RGRP-specific antisense on the signaling and surface expression of the OB-R

HeLa cells were first cotransfected with the expression vectors for OB-R_l and the reporter gene for STAT3, and then with the antisenses. Leptin causes an approximately 1.5-fold increase in the basal activation of the reporter gene for STAT3 in the control cells without antisense, or with a control antisense (AS16) (Fig. 12). In the cells transfected with the antisense specific for OB-RGRP (AS-14), the basal and leptin-stimulated signaling is relatively increased compared to the control conditions. This shows that activation of the JAK/STAT pathway is increased in the cells exhibiting a decrease in OB-RGRP expression. These observations may be explained by an inhibitory effect of OB-RGRP on the basal and OB-R-stimulated activity and, in this case, OB-RGRP can be considered to be a regulator of OB-R signaling. Another alternative is that OB-RGRP might regulate the expression of the surface receptors by limiting the number of OB-R reaching the cell surface. This is in agreement with the fact that only 10 to 20% of the receptors expressed reach the cell surface. In this hypothesis, the decrease in OB-RGRP expression should increase the number of receptors at the cell surface, which should increase the signaling by these receptors. To test this hypothesis, we quantified the number of OB-R_l and OB-R_s receptors expressed at the cell surface in the presence (control) and absence (AS-14) of OB-RGRP (Fig. 13). Transfection of the random antisense showed no effect on the number of receptors expressed at the cell surface, whereas that of the specific antisense (AS-14) caused a 3-fold increase in the number of OB-R expressed at the plasma membrane. Similar results were obtained in nontransfected HeLa cells expressing endogenous receptors. Under these experimental conditions, the total number of receptors, measured by ^{125}I-leptin-binding experiments, showed no significant variations.

All our results are consistent with the role of OB-RGRP in yeast, in protein transport. The increase in surface expression of OB-R appears to be

involved in the increase in signaling observed. However, we cannot entirely exclude the hypothesis that OB-RGRP directly regulates OB-R activity. The application of specific antisenses directed against OB-RGRP should be useful for increasing OB-R signaling in leptin-related disorders, such as human obesity, in which resistance to leptin is observed, characterized by an unadapted response to this hormone. The increase in expression of the receptors at the cell surface and in their signaling should be important for increasing the response to leptin in the case of human obesity, firstly by increasing leptin transport to the brain across the blood-brain barrier and, secondly, by increasing OB-R signaling in the hypothalamus.

The interaction between OB-RGRP and OB-R_s implies that the action of OB-RGRP takes place via this direct interaction with the receptors and that preventing this interaction may lead to the effects of the specific antisense ODN being reproduced. We propose using the BRET test of the interactions between OB-RGRP and OB-R_s, and MYO47 and OB-R_s, described above, as a test for screening molecules which may modulate this interaction. This test may be carried out either on whole or permeabilized cells coexpressing the proteins from fusions of the OB-RGRP and OB-R_s, or MYO47 and OB-R_s BRET partners, or on membrane fractions derived from these cells.